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Modulation of the Xanthine Oxidase/Xanthine Dehydrogenase Ratio by Reaction of Malondialdehyde with NH_2-Groups

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Dedicated to Professor Dr. Dr. Helmut Greiling on the occasion of his 65th birthday

Summary: The potential of xanthine oxidoreductase to generate oxygen radicals depends on the ratio of xanthine dehydrogenase and xanthine oxidase.
Previous studies showed that the lipid peroxidation products, malondialdehyde and 4-hydroxynonenal have different effects on xanthine oxidoreductase activity. These results suggest that the activity of xanthine oxidase, but not xanthine dehydrogenase, is influenced by NH_2-group modulation. We therefore investigated the influence of malondialdehyde on xanthine oxidoreductase. Malondialdehyde reacted with NH_2-groups to form *Schiff* bases, and this reaction was associated with inhibition of xanthine oxidase; SH-groups were not affected. Malondialdehyde had no influence on the xanthine dehydrogenase activity. The inhibited xanthine oxidase was converted to an active xanthine dehydrogenase by dithiothreitol treatment. These experiments indicate the importance of NH_2-groups for xanthine oxidase but not for xanthine dehydrogenase activity. Beside the well known regulation of the xanthine dehydrogenase/xanthine oxidase ratio by the redox status of SH-groups, substances reacting with NH_2-groups of the xanthine oxidoreductase are also able to change the xanthine dehydrogenase/xanthine oxidase activity ratio, thereby influencing the potential to generate oxygen radicals by xanthine oxidoreductase.

Introduction

Xanthine oxidoreductase oxidizes oxypurines (hypoxanthine, xanthine) to uric acid. Under physiological conditions this enzyme acts as a dehydrogenase. Experimental conditions, e. g. ischaemia, hypoxia, reperfusion, lead to the conversion of the xanthine dehydrogenase to an oxidase form (xanthine oxidase) of the enzyme (1), associated with the formation of oxygen radicals, as demonstrated in humans (2–4), rats (2, 5), guinea pigs (3), dogs (6), and other species (7). The relevance of xanthine oxidoreductase as an oxygen radical source (depending on the xanthine dehydrogenase/xanthine oxidase ratio) under in vivo conditions (8–12) is presently under discussion, together with questions concerning the mechanism of the modulation of the xanthine dehydrogenase/xanthine oxidase ratio (13). Recently, we demonstrated that the xanthine dehydrogenase and xanthine oxidase activities of xanthine oxidoreductase are affected differently by the lipid peroxidation product, malondialdehyde, which causes inhibition of xanthine oxidase, leading to an increase of the xanthine dehydrogenase/xanthine oxidase quotient (14). But the mechanism of the malondialdehyde-induced modulation of xanthine oxidoreductase activity is unknown. Since malondialdehyde reacts preferentially with NH_2-groups, we assume that NH_2-groups are important for the oxidase activity but not for the dehydrogenase activity of xanthine oxidoreductase. To test this hypothesis, we analysed

1. the reaction of malondialdehyde with NH_2- and SH-groups in the xanthine oxidoreductase,

2. the xanthine dehydrogenase and xanthine oxidase activities of the dithiothreitol-treated enzyme after incubation with malondialdehyde and

3. the ability to reactivate malondialdehyde-modified xanthine oxidoreductase by dithiothreitol treatment.

Materials and Methods

Pterine, isoxanthopterin, NAD, dithiothreitol and methylene blue were purchased from Sigma (USA), milk xanthine oxidase from Boehringer Mannheim (Germany), malondialdehyde-bis(diethylacetal) from Merck-Schuchardt, Hohenbrunn bei München (Germany) and GSH from Merck, Darmstadt (Germany). All other chemicals used were from commercial sources.

Enzyme preparation

According to *Kaminski & Jezewska* (15), rat liver (male Wistar rats, 200–220 g) was homogenized in 4 vol. 150 mmol/l sucrose, 100 mmol/l Tris/HCl buffer at pH 7.4 with an Ultra Turrax (4 × 30 s, 20 000 min^{-1} at 4 °C). The homogenate was centrifuged for 10 min at 27 000 *g* in a Sorvall RC2-B superspeed centrifuge at 4 °C, then for 60 min at 160 000 *g* in a Beckman L8-55 centrifuge. Ammonium sulphate (1.4 mol/l) was dissolved in the supernatant, which was then centrifuged for 10 min at 15 000 *g*. The ammonium sulphate concentration was increased to 2.4 mol/l, the centrifugation repeated, and the precipitate from this second step dissolved in 50 mmol/l Tris/HCl buffer pH 8.0. Total enzyme activity was 114 ± 2 U/l (about 10 g/l protein; n = 5), consisting of 60 ± 10 U/l (53.2 ± 2.5%) xanthine dehydrogenase and 53 ± 10 U/l (46.8 ± 2.5%) xanthine oxidase. To obtain exclusively xanthine oxidase activity, the enzyme preparation was oxidized with air (12 h, 4 °C).

Malondialdehyde solution

Malondialdehyde was prepared according to *Esterbauer* et al. (16) and *Haberland* et al. (17). Malondialdehyde-bis(diethylacetal)-solution (10 mmol/l) was incubated in 102 mmol/l H_2SO_4 for 60 min at room temperature. After acid hydrolysis, the pH was adjusted to 6.5 using 10 mol/l NaOH. The final concentration was estimated spectrophotometrically at 245 nm (ε = 1375 m^2/mol).

Xanthine dehydrogenase and xanthine oxidase measurement

Xanthine dehydrogenase and xanthine oxidase activity were determined with the fluorimetric method of *Beckman* et al. (18). The activity of xanthine dehydrogenase was measured by detection of isoxanthopterin formation from 0.01 mmol/l pterine. For the xanthine dehydrogenase determination, 0.15 mmol/l NAD was added. Treated or untreated xanthine oxidoreductase preparation (0.05 ml) was added (diluted 1 : 10 with measuring buffer, 50 mmol/l Tris/HCl, pH 8.0). The total volume was 1.0 ml. The measurements were carried out in the Perkin Elmer Fluorimeter MPF-3L (Recorder Perkin Elmer R-100) at 345 nm exitation and 390 nm emission at room temperature.

To obtain specific activities, the spectrophotometric method according to *Kaminski & Jezewska* (15) was used. Xanthine oxidase activity was determined by measuring the absorption increase at 302 nm due to the generation of uric acid. Xanthine dehydrogenase activity was determined by measuring the absorption increase at 340 nm due to the generation of NADH. The measuring cuvette contained the sample, 0.05 mmol/l xanthine, 0.15 mmol/l NAD (for xanthine dehydrogenase estimation only) and 50 mmol/l Tris/HCl buffer, pH 8.0 in a final volume of 2.5 ml.

Determination of the content of SH-groups

For the determination of the content of SH-groups, a procedure according to *Sedlak & Lindsay* (19) was used. Malondialdehyde-treated xanthine oxidoreductase preparation (0.1 ml) was incubated with 0.15 ml 100 mmol/l Tris, pH 8.2, 0.01 ml 10 mmol/l 5,5' dithio-bis (2-nitrobenzoic acid) and 1.24 ml 18 mol/l sodium dodecyl sulphate for 1 h at room temperature. The absorbance was measured at 405 nm with a Carl Zeiss Jena Spectrophotometer M40.

Determination of the content of *Schiff* bases

For the estimation of *Schiff* bases, a modification of the method of *Quehenberger* et al. (20) was used. Malondialdehyde-treated xanthine oxidoreductase preparation (protein content about 1.0 g/l) (1.0 ml) was mixed with 1.0 ml 612 mmol/l trichloroacetic acid solution. The precipitate was removed by centrifugation (10 min, 1500 *g*), washed, then dissolved in 1.0 ml of a 104 mmol/l sodium dodecyl sulphate solution. The fluorescence intensity of the *Schiff* bases was measured with the Perkin Elmer Fluorescence Spectrometer 3000 at an excitation wavelength of 400 nm and an emission wavelength of 460 nm.

Values are expressed as mean ± SD.

Results

The investigations were carried out with xanthine oxidoreductase prepared from rat liver. The preparation possessed xanthine dehydrogenase and xanthine oxidase activities, or only xanthine oxidase activity after air oxidation of the xanthine dehydrogenase. During air oxidation, the total activity of xanthine oxidoreductase remained unchanged (data not shown). Table 1 demonstrates a time-dependent loss of the xanthine oxidase activity during incubation of the oxidized xanthine oxidoreductase with malondialdehyde. A continuous formation of *Schiff* bases was detectable, whereas the SH-group content of the xanthine oxidoreductase was not changed during malondialdehyde treatment. Xanthine oxidoreductase prepared by reconversion of an exclusively xanthine oxidase-containing preparation by dithiothreitol treatment (a representative preparation containing 40% xanthine dehydrogenase and 60% xanthine oxidase was used), showed that only the xanthine oxidase activity was influenced by malondialdehyde, whereas the reconverted xanthine dehydrogenase remained unchanged (fig. 1). Incubation of the malondialdehyde-in-

Tab. 1 Xanthine oxidase activity, *Schiff* base formation (F = fluorescence intensity) and SH-group content during incubation of xanthine oxidoreductase (60 U/l xanthine dehydrogenase, 53 U/l xanthine oxidase) with 1 mmol/l malondialdehyde (n = 4).

Incubation time (min)	Xanthine oxidase activity (%)	*Schiff* bases (F)	SH-groups (%)
0	100	128.5 ± 0.7	100
30	53 ± 7.0	149.5 ± 4.2	100
60	46 ± 7.7	162.5 ± 0.7	100
90	21 ± 8.3	189.5 ± 23.3	100

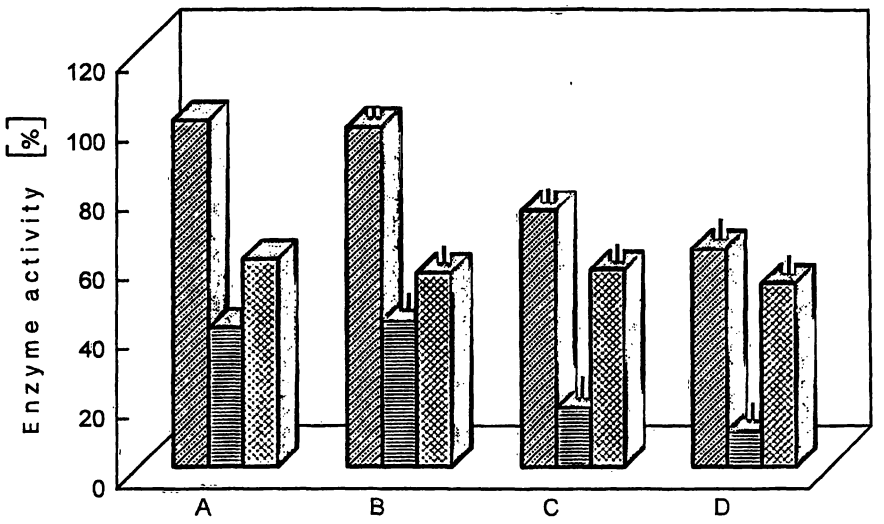

Fig. 1 Influence of malondialdehyde on xanthine oxidoreductase (60% xanthine dehydrogenase, 40% xanthine oxidase) prepared by partial reconversion of xanthine oxidase by 30 min dithiothreitol treatment (n = 4).
(10 U/l xanthine oxidase; 10 mmol/l dithiothreitol; 1 mmol/l malondialdehyde; pH 6.5)
In the control, xanthine dehydrogenase/xanthine oxidase was incubated in the solvent used in the malondialdehyde preparation.

▨ xanthine oxidase + xanthine dehydrogenase
▤ xanthine oxidase
▩ xanthine dehydrogenase
A: xanthine dehydrogenase/xanthine oxidase
B: 60 min incubation of xanthine dehydrogenase/xanthine oxidase in the malondialdehyde solvent (control)
C: 30 min incubation of xanthine dehydrogenase/xanthine oxidase with malondialdehyde
D: 60 min incubation of xanthine dehydrogenase/xanthine oxidase with malondialdehyde

activated xanthine oxidase with dithiothreitol resulted in an increase of the total xanthine oxidoreductase activity and a partial reconversion of xanthine oxidase to xanthine dehydrogenase. This is shown by the two experiments illustrated in figures 2a and 2b, using a xanthine oxidoreductase preparation containing only xanthine oxidase. Figure 2a demonstrates that 30 min of malondialdehyde treatment decreased the pure xanthine oxidase-containing preparation to 55% of its initial activity. A subsequent dithiothreitol treatment of 60 min led to an increase of the total activity to 75% of the initial level. After this dithiothreitol treatment, the total activity consisted of 24% xanthine oxidase and 76% xanthine dehydrogenase. The same xanthine oxidase preparation was used in fig. 2b. Xanthine oxidase treated with malondialdehyde for 180 min (residual xanthine oxidase activity of 20%) was also reactivated by dithiothreitol (total activity of 53% of the initial level) and reconverted to xanthine oxidoreductase containing 21% xanthine oxidase and 72% xanthine dehydrogenase (fig. 2b). The xanthine dehydrogenase obtained by dithiothreitol reconversion of malondialdehyde-modified xanthine oxidase showed a higher heat stability than untreated xanthine dehydrogenase. Also, a commercial preparation of xanthine oxidase was similarly inhibited by malondialdehyde, but neither reactivation nor reconversion were possible (fig. 3); the malondialdehyde-induced inhibition was stronger than that observed for xanthine oxidase prepared from liver, and it continued during dithiothreitol incubation.

Discussion

The formation of oxygen radicals by the oxidase activity (xanthine oxidase) of xanthine oxidoreductase is regarded as one cause of lipid peroxidation and other cytotoxic reactions in biological systems. Recently, we reported the different influences of lipid peroxidation products on the xanthine dehydrogenase and xanthine oxidase activity of xanthine oxidoreductase. Since malondialdehyde inhibited the xanthine oxidase, it appeared that the lipid peroxidation product, malondialdehyde, might influence the ability of xanthine oxidoreductase to generate oxygen radicals, a process in which malondialdehyde would act as a feedback inhibitor. Malondialdehyde is a typical NH_2-group reagent (21, 22). Therefore reactions of malondialdehyde with NH_2-groups might be responsible for the effect of malondialdehyde on xanthine oxidoreductase.

Xanthine oxidoreductase was prepared from rat liver and oxidized by air to obtain exclusively xanthine oxidase, with no xanthine dehydrogenase activity. This procedure was based on a report of *Parks & Granger* (23). In accordance with *Della Corte* et al. (24), our experiments demonstrating the conversion of xanthine dehydrogenase to xanthine oxidase by oxidation, and the reconversion by dithiothreitol show that the ratio of xanthine dehydrogenase and xanthine oxidase activities in the xanthine oxidoreductase depends on the redox status of the SH-groups. Whereas SH-group oxidation led only to the conversion of xanthine dehydrogenase to xanthine

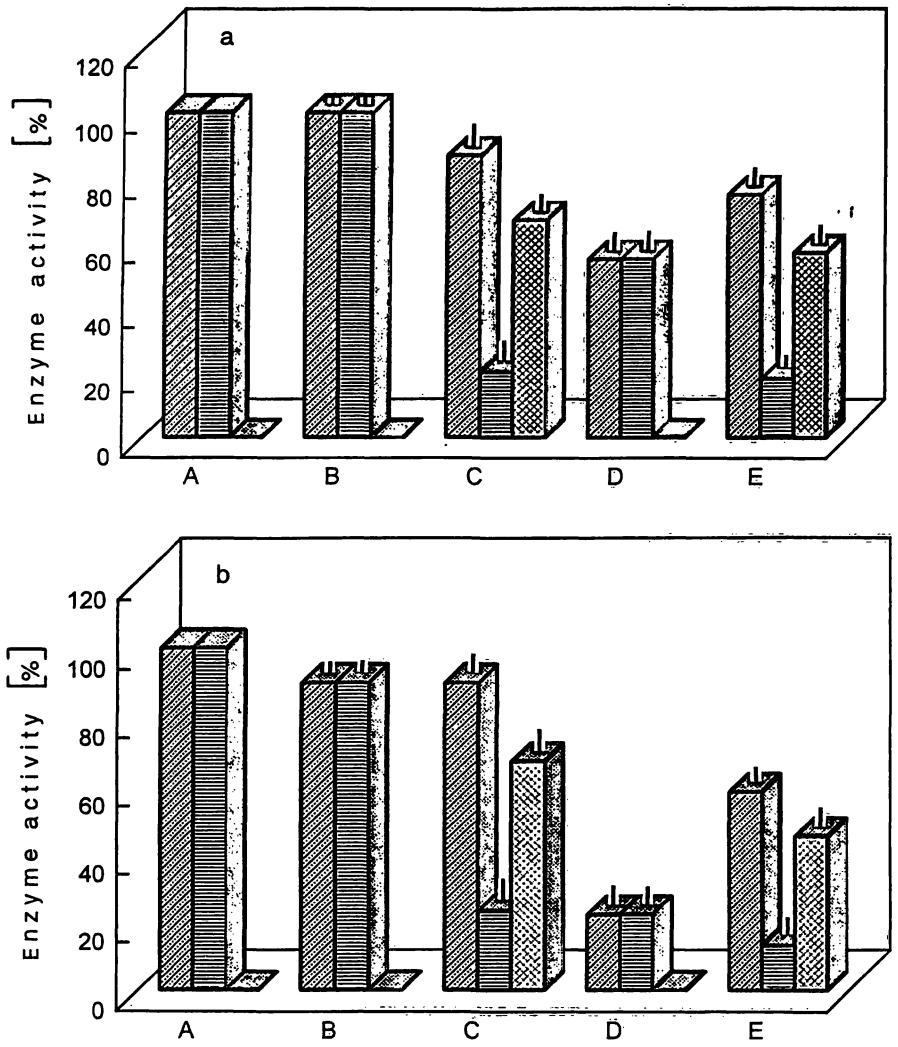

Fig. 2 Influence of malondialdehyde on xanthine oxidase, prepared by air oxidation of rat liver xanthine oxidoreductase followed by incubation with dithiothreitol (n = 4).
(10 U/l xanthine oxidase; 1 mmol/l malondialdehyde; 10 mmol/l dithiothreitol, pH 6.5)
In the control, xanthine oxidase was incubated in the solvent used in the malondialdehyde preparation.

▨ xanthine oxidase + xanthine dehydrogenase

▤ xanthine oxidase

▩ xanthine dehydrogenase

a) A: xanthine oxidase
B: 30 min incubation of xanthine oxidase in the malondialdehyde solvent (control)
C: 30 min incubation of xanthine oxidase in the malondialdehyde solvent followed by 60 min incubation with dithiothreitol
D: 30 min incubation of xanthine oxidase with malondialdehyde
E: 30 min incubation of xanthine oxidase with malondialdehyde followed by 60 min incubation with dithiothreitol

b) A: xanthine oxidase
B: 180 min incubation of xanthine oxidase in the malondialdehyde solvent (control)
C: 180 min incubation of xanthine oxidase in the malondialdehyde solvent followed by 90 min incubation with dithiothreitol
D: 180 min incubation of xanthine oxidase with malondialdehyde
E: 180 min incubation of xanthine oxidase with malondialdehyde followed by 90 min incubation with dithiothreitol

oxidase without alteration of the total activity of xanthine oxidoreductase (previously observed (25, 26)), malondialdehyde treatment resulted in a decrease of the total activity. Based on the continuous formation of *Schiff* bases during the incubation of xanthine oxidoreductase with malondialdehyde and the unchanged content of SH-groups under these conditions, we conclude that the reaction of malondialdehyde with NH_2-groups in the xanthine oxidase is responsible for the activity decrease and that free NH_2-groups in the xanthine oxidoreductase are necessary for the xanthine oxidase activity. This is supported by the experiments using commercial xanthine oxidase, which could also be inhibited by malondialdehyde treatment. In contrast to the malondialdehyde effect on xanthine oxidase, the xanthine dehydrogenase activity was not influenced by malondialdehyde treatment. On the other hand, malondialdehyde-inhibited xanthine oxidase was reconverted to an active xanthine dehydrogenase with dithiothreitol. Therefore, we assume that the NH_2-groups in the xanthine oxidoreductase have

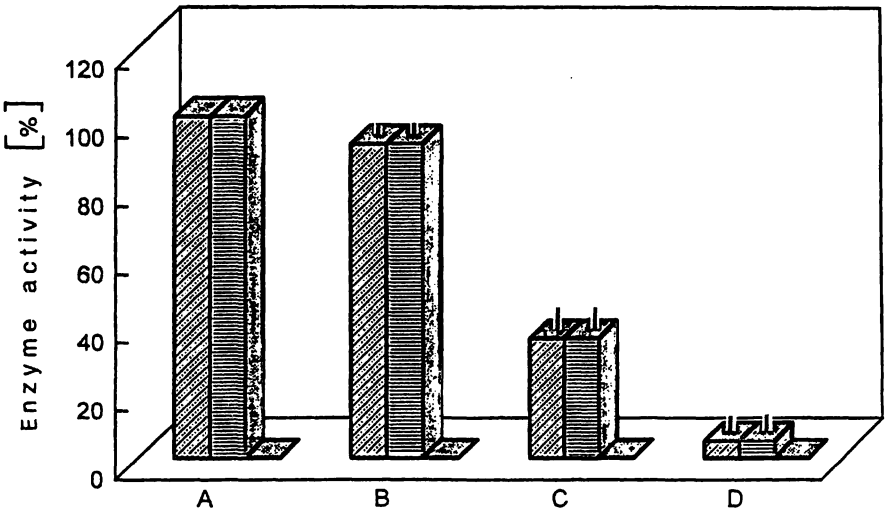

Fig. 3 Influence of malondialdehyde on commercial milk xanthine oxidase (Boehringer Mannheim) followed by incubation with dithiothreitol (n = 4).
(10 U/l xanthine oxidase; 1 mmol/l malondialdehyde; 10 mmol/l dithiothreitol; pH 6.5)
In the control, xanthine oxidase was incubated in the solvent used in the malondialdehyde preparation.

▨ xanthine oxidase + xanthine dehydrogenase

▤ xanthine oxidase

▩ xanthine dehydrogenase

A: xanthine oxidase

B: 30 min incubation of xanthine oxidase in the malondialdehyde solvent followed by 60 min incubation with dithiothreitol (control)

C: 30 min incubation of xanthine oxidase with malondialdehyde

D: 30 min incubation of xanthine oxidase with malondialdehyde followed by 60 min incubation with dithiothreitol

no or only little influence on the xanthine dehydrogenase activity. It was not surprising that the malondialdehyde-inhibited commercial xanthine oxidoreductase could not be restored by dithiothreitol treatment. Preparation of commercial xanthine oxidase includes a proteolytic step (27) and this enzyme is not reconvertible by disulphide group reduction (25).

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